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Carrier-Mediated Antiviral Therapy

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I. INTRODUCTION

Drug-delivery systems that appear to be suitable for antiviral compounds can be grouped in three major categories (Table 1). Accordingly, when a drug is encapsulated in a carrier or attached to macromolecules, endocytosis is the only mode of entry. Two types of endocytosis, phagocytosis and pinocytosis, constitute the physiologic basis of the delivery systems. In phagocytosis, particulate materials are transported in large intracellular vesicles. The drug is encapsulated in an insoluble carrier engulfed by phagocytic cells and is released after enzymatic breakdown of the carrier. In pinocytosis, soluble materials are transported in small vesicles. The linkage between drug and soluble carrier is stable in the plasma but is susceptible to hydrolysis by lysosomal enzymes. The third is an in-between type. The substance, the drug or the biological is encapsulated in a polymeric or liposomal carrier that, because of its location or size, is not taken up by the cells. Because of diffusion of the substance and bioerosion of the carrier, the biological or the drug is constantly released from the carrier into the circulation and eventually enters the cell via active or passive transport.

II. DRUG TRANSPORT VIA ENDOCYTIC PHAGOCYTOSIS

A. Treatment of Rift Valley Fever Virus Infection with Liposome-Encapsulated Ribavirin

The best-known example of drug transport via phagocytosis involves liposomes, which are spontaneously formed phospholipid vesicles.

Three types of liposomes can be produced based on the method of preparation:

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Table 1. Systems for Targeted and/or Prolonged Delivery of Antiviral Compounds

Drugs encapsulated or attached to macromolecular carrier enter cells via:		
Endocytic phagocytosis	Endocytic pinocytosis	Active or passive transport
Particulate materials transported in large intracellular vesicles	*Soluble* materials transported in small vesicles	Spherical, circulating, or localized reservoir
Drug encapsulated in an insoluble carrier engulfed by phagocytic cells	Linkage between drug and soluble carrier stable in the plasma but is cleaved in the cell by enzyme(s)	Release of the incorporated substance controlled by diffusion and erosion
Drug released after enzymatic breakdown of the carrier		Free drug enters the cell by active or passive transport

1. Small unilamellar vesicles (SUV) with a diameter of 30 μm are prepared by sonication. SUV of 30–110 μm are formed when an ethanol solution of the lipid is injected into an aqueous phase solution. A third method of producing SUV is to pass multilamellar vesicles (MLV) through a French press with a resulting size range of 30–50 μm.
2. Large unilamellar vesicles (LUV) are formed with reverse-phase evaporation and with detergents. The molar ratio of the lipid/detergent determines the size of the liposomes, which ranges from 50 to 10,000 μm.
3. Multilamellar vesicles with a size range of 400–7000 μm are prepared by forming a lipid film in a pear-shaped flask by using a rotary evaporator. The drug-encapsulation efficacy is best with LUV and MLV but is also dependent on the drug.

The size of the liposome is believed to have an impact on the uptake by cells in different organs. Inclusion of a targeting ligand further promotes targeting into specific cells. Liposomal encapsulation is frequently used to deliver drugs to macrophages infected with fungi, bacteria, and parasites (Alving, 1983; Alving *et al.*, 1978; Desiderio and Campbell, 1983; Graybill *et al.*, 1982; Lopez-Berestein *et al.*, 1983; New *et al.*, 1981; Tremblay *et al.*, 1984). The best result was obtained with an antileishmanial drug: treatment of experimental leishmanias required about 700 times less liposome-encapsulated drugs than did free drugs (Alving, 1983; Alving *et al.*, 1978).

Encapsulation of ribavirin into MLV increased the amount of drug in various organs. When compared to treatment with free drug, four times more drug was delivered into the liver, 25 times more to the spleen, and 10 times more into the lung (Kende *et al.*, 1985) (Fig. 1). Figure 2 shows the increased therapeutic efficacy of liposome-encapsulated ribavirin against Rift Valley Fever virus (RVFV) infection in mice.

B. Treatment of Herpes Simplex Virus Type 1 Infection with Liposome-Encapsulated Ribavirin or Muramyl Tripeptide

A study at the University of South Carolina examined the therapeutic advantage of liposomal encapsulation of ribavirin and muramyl tripeptide-phosphatidyl ethanolamine

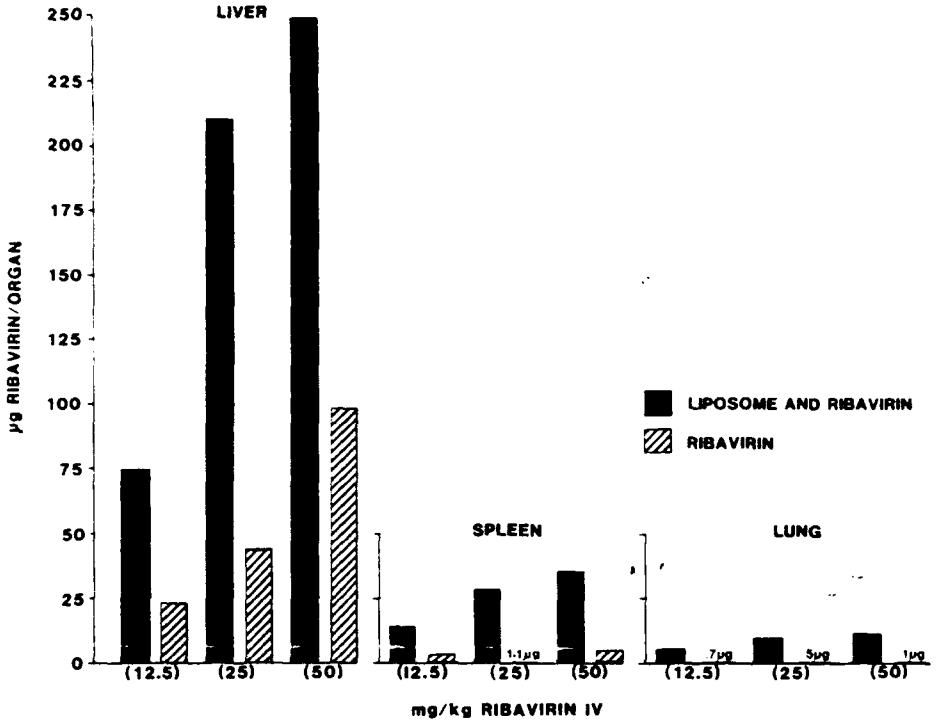

Figure 1. Distribution of [^{14}C]ribavirin, with or without liposomes in mouse tissue 1 hr after IV injection.

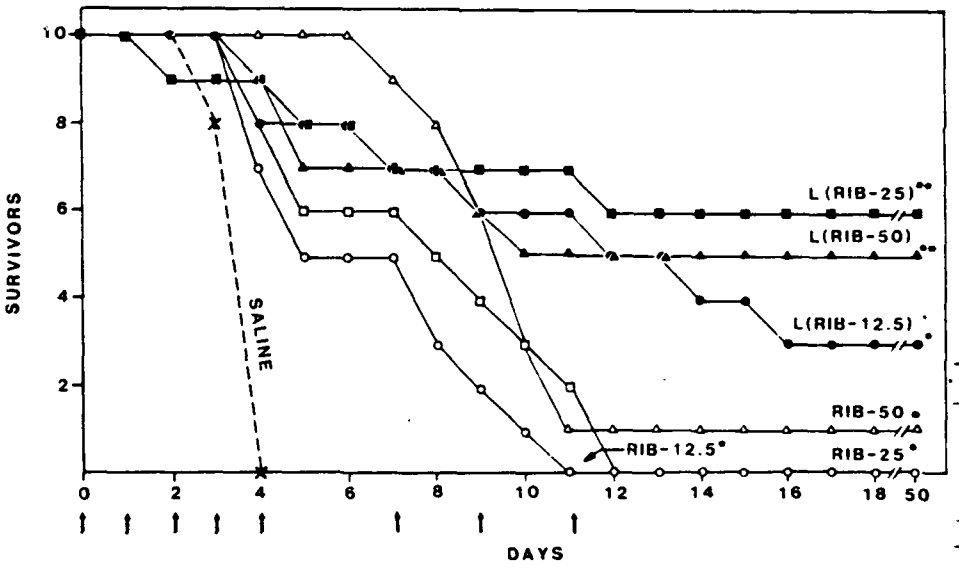

Figure 2. Efficacy of liposome-encapsulated ribavirin (L-RIB) against Rift Valley fever virus infection (day 0) in Swiss Webster mice ($N = 10$). Arrows indicate the days of treatment. Open symbols: 12.5, 25, and 50 mg/kg ribavirin (RIB). Filled symbols: 12.5, 25, and 50 mg/kg L(-RIB). *$p > 0.001$: not significant; **$p < 0.01$: significant in comparison with placebo treatment. The p value of all L-RIB-treated mice versus RIB-treated mice was 0.0001.

(MTP-PE) in the treatment of viral diseases involving the lung, liver, or central nervous system (CNS) as the primary sites of viral infection. Both compounds appear to be potentially useful chemotherapeutic agents for the treatment of human viral diseases (Sidwell *et al.*, 1973; Fidler *et al.*, 1982; Gisler *et al.*, 1982); however, the intravenous dosages required to maintain effective drug levels in infected organs, such as the lung or liver, may adversely affect the normally safe therapeutic indices of these drugs.

The murine models of HSV-1 infection were selected for study for several reasons:

1. Infection of weanling mice is followed by a high death rate, and the pathology is similar to that observed in human disease (Nachtigal and Caulfield, 1984; De Clercq and Luczak, 1976).
2. Ribavirin has been shown to inhibit the replication of both viruses (Sidwell *et al.*, 1973; Durr *et al.*, 1975) but is considerably more effective against influenza.
3. Activation of tissue macrophages by MTP-PE has been shown by others to enhance resistance to HSV-2 infection (Koff *et al.*, 1985; Dietrich *et al.*, 1983).

Negatively charged MLV were used as carrier vehicles for both ribavirin and MTP-PE (Kende *et al.*, 1985; Koff *et al.*, 1984). By using MLV as carrier vehicles, encapsulation efficiencies of 20% for ribavirin and >90% for lipophilic MTP-PE were achieved. In efficacy studies, liposome-encapsulated ribavirin was more effective than free ribavirin in enhancing survival (Fig. 3); moreover, the ribavirin dosage used in liposome carriers (2 mg per mouse) was only one-fifth that used in free form (10 mg per mouse).

The effect provided by liposomal encapsulation on the antiviral efficacy of MTP-PE was examined in three models of HSV-1 disease in which different organs serve as

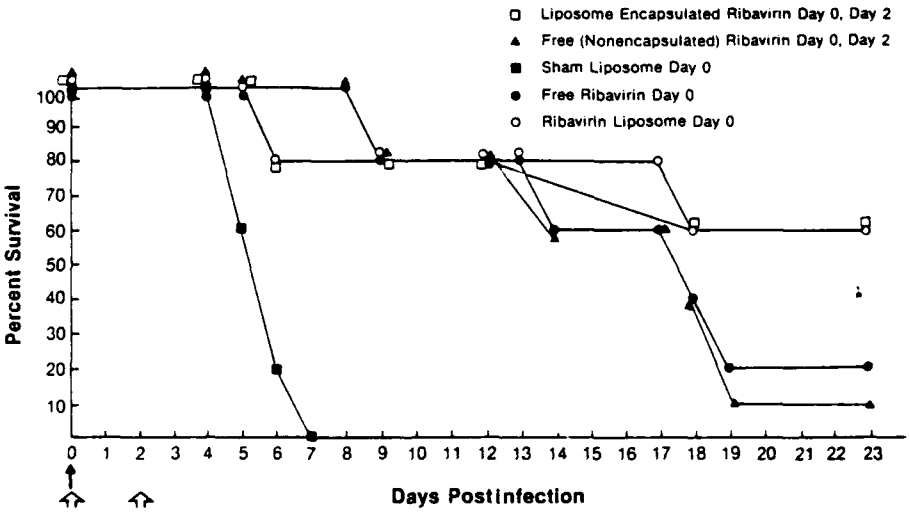

Figure 3. Ribavirin treatment of influenza virus-infected mice. Mice (N = 10) were inoculated intranasally with 10 LD_{50} of influenza virus and treated intravenously with either free (10 mg per mouse) or liposome-encapsulated ribavirin (3 mg per mouse) on days 0 and 2.

Table 2. Effect of MTP-PE Treatment on the Survival of Mice Infected Intranasally on Day 0 with HSV-1

Treatment groups	Treatment schedule[b]	Survivors		% Survival	Mean survival
		Day 8	Day 23	Day 23	time
Uninoculated controls	—	0/20	0/20	0	5.10
Sham liposome	−3, 0, 2	7/20	3/20	15	7.35
Free MTP-PE[a]	−3, 0, 2	10/20	9/20	45[c]	8.09[c]
Free MTP-PE[a]	0, 2	9/20	6/20	30[a]	8.75[c]
Liposome-encapsulated MTP-PE[a]	−3, 0, 2	18/20	16/20	80[c]	9.50[c]
Liposome-encapsulated MTP-PE[a]	0, 2	14/20	8/20	40[c]	8.25[c]

[a] 100 μg per mouse.
[b] Days pre- and postinfection, IV drug administration.
[c] $p < 0.001$ compared with uninoculated control group.

primary sites for viral replication: (1) intranasal instillation resulted in interstitial pneumonia and adrenal involvement, (2) footpad inoculation resulted in viral passage through the sciatic nerve to the spinal cord and brain, and (3) intravenous (IV) inoculation resulted in a generalized infection where the liver appeared to be the primary site of viral replication; but the lungs, spleen, adrenals, and CNS were also involved late in the course of infection.

Table 2 illustrates the effect of IV MTP-PE on the survival of mice injected intranasally with HSV-1. A small but significant enhancement of survival was observed when either free or liposome-encapsulated MTP-PE was administered on the day of inoculation and 2 days postinfection. More dramatic protection was observed when an additional dose of MTP-PE was administered 3 days prior to infection. Moreover, liposome-encapsulated MTP-PE was superior to free MTP-PE.

The enhanced survival observed after MTP-PE treatment was correlated with a reduction in viral titers in both the lung and adrenal glands (Fig. 4). MTP-PE-treated survivors had elevated neutralizing antibody titers and were resistant to viral rechallenge 21 days after the initial infection (Table 3).

By contrast, mice were not protected from the IV administration of virus, which leads to a more generalized infection. Neither liposome-encapsulated nor free MTP-PE, given several days following infection, protected mice from IV HSV-1 challenge (data not shown). Experiments were performed in which intranasal and IV routes of MTP-PE administration were combined. Figure 5 illustrates that combined routes of drug administration were highly effective in protecting mice from lethal IV HSV-1 challenge. The therapeutic activity of intranasally administered MTP-PE on a pure CNS disease induced by footpad inoculation of HSV-1 is illustrated in Fig. 6: free MTP-PE given intranasally was more effective than liposome-encapsulated MTP-PE. In addition, mice receiving free MTP-PE had lower viral titers in their spinal cords (Fig. 7).

In combination studies, single doses of MTP-PE on day 3, or ribavirin on day 0, had little effect on survival; however, when given together, 80% of the animals were protected (Table 4). These data suggest that when a virus infects an organ that is part of the reticuloendothelial system (RES), liposomes can be used to enhance the therapeutic index

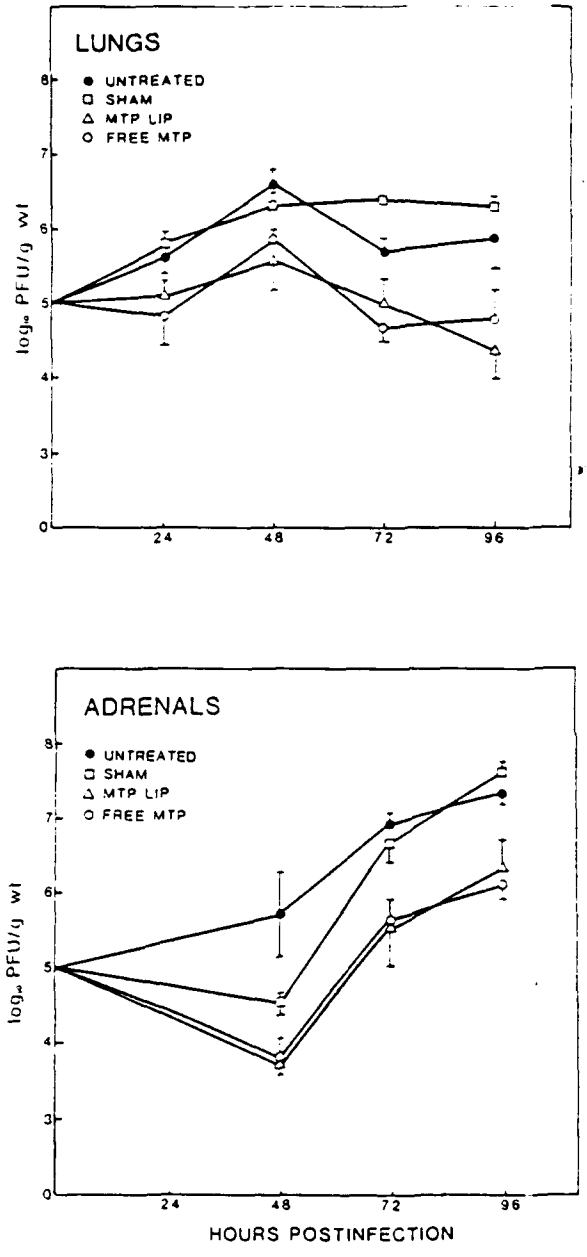

Figure 4. Inhibition of virus replication. Herpes simplex virus type 1 (HSV-1) titers in lungs and adrenals following MTP-PE treatment; 4- to 5-week-old mice were inoculated intravenously with 0.2 ml of either sham, liposome-free, or liposome-encapsulated MTP-PE (100 μg/day) prior to, on the day of, and 3 days following a challenge with 10 LD_{50} of HSV-1. Virus in cell-free extracts of lungs and adrenals was titrated on Vero cells ($N = 3$).

Table 3. Antibody Response of Mice to HSV-1 Infection in MTP-PE-Treated Survivors[a]

Titer	Number of animals with serum-neutralizing titers[b]	
	Free MTP	MTP-PE LIP
<40	3	0
40–100	4	2
100–200	3	3
500–1000	2	8
1000–2500	5	6
Mean titer	489	840

[a] MTP-PE, muramyl tripeptide-phosphatidyl ethanolamine; LIP, liposome.
[b] $p = 0.09$ by Wilcoxon's ranking test, when free MTP-PE and MTP-PE LIP groups were compared.

of selected antiviral agents. This enhancement probably results from macrophage trapping and localization. By contrast, the enhanced resistance observed in the HSV-1 encephalitis model appears to be due to the ease with which free MTP-PE can cross the blood–brain barrier (BBB) following intranasal inoculation. Nonetheless, liposomes may provide a unique means by which drug combinations (e.g., immunostimulants and antivirals) can be

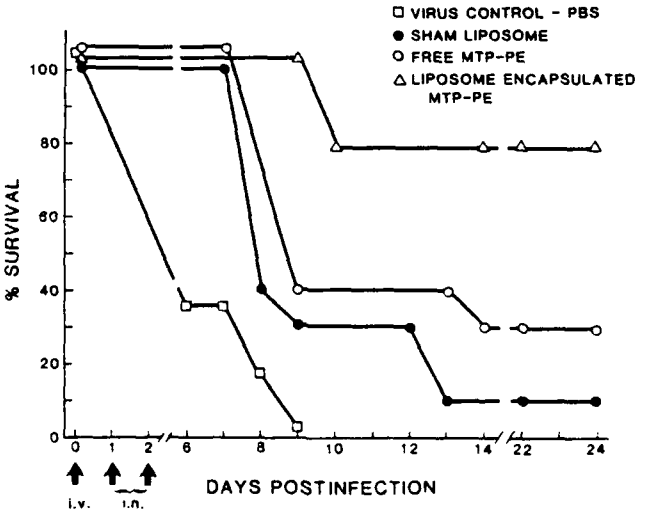

Figure 5. Therapeutic value of MTP-PE in disseminated herpes simplex virus type 1 (HSV-1) infections. Mice were infected IV with 10^4 PFU of the virus and treated on day 0 with 100 μg (IV) of either liposome-encapsulated or free MTP-PE. This was followed by the intranasal administration of 100 μg free MTP-PE on days 1 and 2 postinfection.

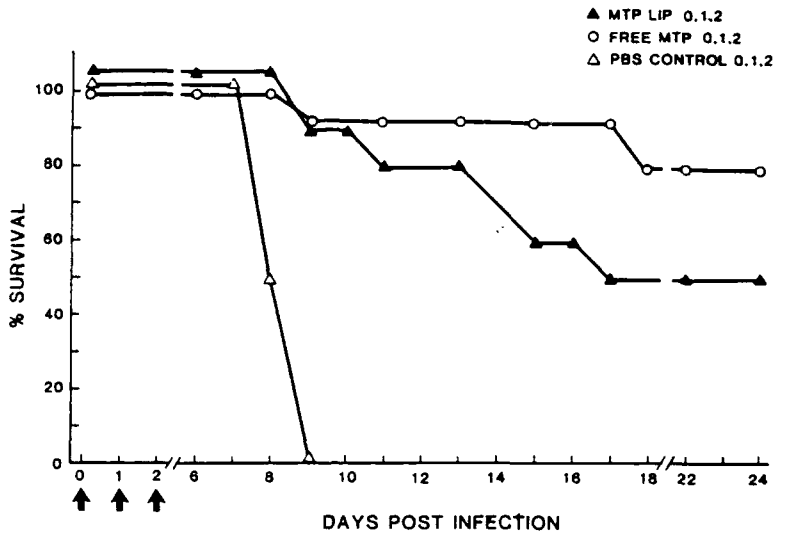

Figure 6. MTP-PE therapy of herpes simplex virus type 1 (HSV-1)-induced encephalitis; 4-week-old mice were inoculated via footpad with 5×10^5 PFU of HSV-1. Either free or liposome-encapsulated MTP-PE (100 μg per mouse) was administered on days 0, 1, and 2 postinfection.

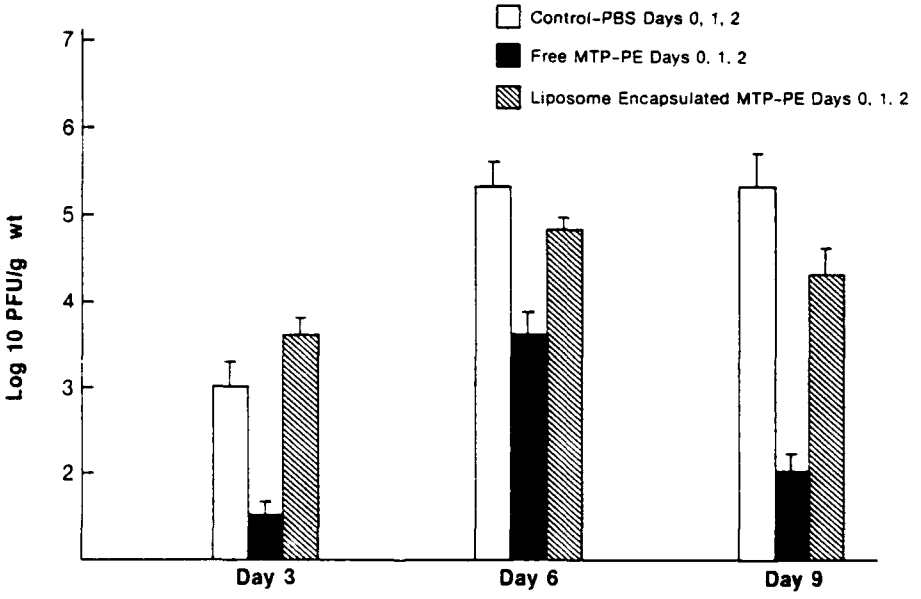

Figure 7. Herpes simplex virus type 1 (HSV-1) viral titers in spinal cords of MTP-PE-treated mice; 4-week-old mice were treated as described in Fig. 5. Spinal cords were removed on days indicated and homogenized, and virus was titrated on Vero cells.

Table 4. Combined Ribavirin/MTP-PE Therapy of HSV-1 Pneumonitis

Treatment groups[a]	Treatment schedule[b]	Survivors		% Survival day 23	Mean survival times[c]
		Day 10	Day 23		
Uninoculated control	—	0/20	0/20	0	7.4
Sham liposome	−3 or 0	0/10	0/10	0	7.6
L-MTP-PE alone	−3	2/10	2/10	20	7.8
L-RIB alone	0	1/10	0/10	0	7.7
L-MTP-PE + L-RIB	−3 and 0	8/10	8/10	80[d]	7.5

[a] MTP-PE (100 μg per mouse); ribavirin (3 mg per mouse).
[b] Days pre- and postinfection, IV drug administration.
[c] Of animals that died.
[d] $p < 0.001$ compared with control group.

combined in the same carrier vehicle and delivered to specific sites of viral replication in a diseased host.

III. DRUG TRANSPORT VIA ENDOCYTIC PINOCYTOSIS

Targeting of Polymer Matrix–Drug Complex into Specific Cells

Hydroxypropylmethacrylamide (HPMA) polymer represents the second type of drug-delivery system (Kopacek *et al.*, 1985), in which the carrier-drug complex enters the cells via pinocytosis. The matrix of the carrier is HPMA, and the drug (*p*-nitroaniline) is attached to the matrix via an enzymatically degradable amino acid spacer.

Two important points must be studied. First, in the blood (serum), the bond between the drug and the carrier should be stable. It was found that the release of *p*-nitroaniline in plasma and serum is negligible with respect to the rate of cleavage by lysosomal enzymes (Kopacek *et al.*, 1985). Second, inside the cell, however, the bond between the drug and the carrier should be cleaved by lysosomal enzymes. These workers showed that the time-dependent cleavage of *p*-nitroaniline from the polymeric substrate was catalyzed by rat liver tritosomes and cathepsin H. Cathepsin B and L did not cleave the drug.

To avoid accumulation of nondegraded polymers, low-molecular-weight poly-HPMA chains can be prepared with cross-linking. Such polymers can be cleaved by chymotrypsin, trypsin, papains, and cathepsin B and L.

To achieve selective targeting of polymer-bound drugs, it is necessary to incorporate a specific residue, such as a glycolipid that interacts with membrane receptors unique to specific cell types. For instance, hepatocytes have membrane receptors that recognize galactose residues, which permit specific targeting of the drug carrier into liver hepatocytes.

Distribution studies in rats with ^{125}I-labeled HPMA water-soluble cross-linked copolymer showed that 60 min after injection, galactosamine-containing HPMA polymer assumes a high (63%) content in the liver, while without targeting, only 6% of labeled polymer is found in the liver. This system has not yet been deployed to deliver antiviral compounds, but it has all the features necessary for drug targeting into specific cells.

IV. DRUG UPTAKE BY ACTIVE OR PASSIVE TRANSPORT

For reservoir type of prolonged and controlled delivery of a substance, localized liposomes, circulating (systemic) polylactic acid, or polylactic/polyglycolic acid copolymer microcapsules have been used. The drug release is controlled by a combination of diffusion of the compound and erosion of carrier.

A. Localized Sustained Release of Interferon by Liposomes

Interferons α and β (IFN_α and IFN_β), produced both by recombinant DNA technology as well as purified from natural sources, have been shown to be efficacious in treating certain cancers and viral diseases. Studies with IFN_γ have more recently been undertaken; thus their clinical value is not yet as well defined. The treatment schedules usually involve multiple injections of the interferon over a period of several weeks. By using such treatment regimens, the doses of the interferons needed to obtain efficacy can result in toxic side effects. For all these reasons, methods of increasing the ease of administration as well as the therapeutic ratio of interferons are warranted.

Sustained-release liposomal–interferon preparations were employed for systemic and/or localized treatment. IFN_β or IFNγ was incorporated with high efficacy (100% for IFN_β, 50–100% for IFN_γ) into lyophilized multilamellar liposomes prepared by hydrating lyophilized lipids with an aqueous solution of the interferon (Eppstein *et al.*, 1985; Eppstein, 1986*a*,*b*; Eppstein and Felgner, 1987).

The presence of serum and cells induces the leakage of the interferon from the liposome preparations, with the most solid vesicles (i.e., those made with phospholipids having saturated acyl chins) being the least leaky. Accordingly, liposome compositions can be prepared to control the rate of release of the interferon to different degrees after subcutaneous (SC) or intramuscular (IM) injection. Formulations of recombinant human $IFN\beta_{ser17}$ ($rHuIFN_{\beta ser17}$, obtained from Tirton Biosciences Inc., Alameda, California) in MLV consisting of diarachidoyl-phosphatidylcholine–dipalmitoylphosphatidylglycerol (DAPC–DPPG), 9 : 1, were able to retain the interferon at the site of IM or SC injection in mice. This resulted in a slow interferon release from the vicinity of the injection site over 1–2 weeks. Therefore, interferon-containing liposomes will not be phagocytized by macrophages, which would result in the enzymatic digestion of interferon. Thus, 50% of the interferon was still retained after 2 days, 15% remained after 6 days, and 5% remained after 9 days. By contrast, free interferon was gone after 1 day (Eppstein, 1986*a*; Eppstein and Felgner, 1987). When saturated phospholipids of progressively shorter acyl chain length (C_{18}, C_{16}, and C_{14}) were employed, the duration of retention of the interferon was progressively reduced (Faulkner, Schryver, and Eppstein, unpublished results).

The efficacy of liposomal formulations of $rHuIFN_{\beta ser17}$ was studied in a primate model of varicella–zoster virus (VZV). African green monkeys were infected systemically with simian VZV, and 24 hr later, interferon treatments were initiated. Initial results indicated that free $rHuIFN_{\beta ser17}$, given IM twice daily at 10^6 U/kg/dose for 10 days (2×10^7 U/kg total dose), showed very good efficacy in suppressing systemic VZV infection. The treated monkeys had minimal viremia and rash, and they did not die. Placebo-treated controls showed extensive viremia and rash and died 10–11 days postinfection. However, the same dosage of interferon was marginally effective when given in

only two or three injections (on days 1 and 6 or on days 1, 4, and 7 postinfection) instead of the 20 twice-daily injections. By contrast, if this same dosage of interferon was given on days 1 and 6, but in a liposomal formulation, distearoylphosphatidylcholine (DSPC)–DPPG (9 : 1), 40 μmoles lipid/kg per dose, intermediate efficacy was obtained; death was prevented and viremia and rash were reduced, although not to the extent obtained with the 20 doses of free interferon (Soike *et al.*, in press). Formation of neutralizing antibodies to the human IFN_β was not obtained in monkeys 1–4 weeks after injection of the second dose of liposomal interferon. These initial results suggest that liposomal interferon formulations can be obtained that will enhance the efficacy of an injection of interferon, most likely by providing a slow release of the drug over several days.

B. Polymeric Microcapsule Carriers for Drugs and Viral Vaccine

Persistent levels of the contraceptive norethisterone hormone were obtained in baboon serum with polylactate microcapsules. The amount of norethisterone is related to size of the capsules. Polylactide–polyglycolide microcapsules containing norethisterone were used in humans to maintain serum levels for 6 months (Beck *et al.*, 1980).

In diabetic rats that received SC implants of insulin containing ethylene vinyl acetate polymer, the blood glucose level was normal for 4 weeks, in contrast to the high glucose level in those rats that received placebo-containing polymer (Creque *et al.*, 1980). Sustained release with ethylene vinyl acetate polymer pellets as carrier and immunologic adjuvant of bovine serum albumin (BSA) has also been described (Langer, 1981). The immune response evoked in rats with a single injection of BSA–polymer was comparable to or better than two injections of BSA alone.

The development of more specific and purer vaccines very often leads to a decrease in the antibody response, hence protection. This effect was observed some years ago during the development of split vaccines (Kreuter and Speiser, 1976*a*; Kreuter *et al.*, 1976) but may be more pronounced during the development of vaccines produced by genetic engineering. This problem may be overcome by the use of adjuvants.

Because of their side effects, very few adjuvants can be used in human vaccines. Besides the classic aluminum adjuvants, particulate polymeric carriers, the so-called nanoparticles, hold promise for use as adjuvants. Nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm (1 μm). They consist of macromolecular materials in which the active principle (antigen or drug) is dissolved, entrapped, or encapsulated or to which the active principle is adsorbed or attached (Kreuter, 1983). These materials are biodegradable (Grislain *et al.*, 1983; Kreuter *et al.*, 1983). The tissue response 1 year after IM injection of poly(methylmethacrylate) nanoparticles was very mild, comparable to that of a fluid vaccine (Kreuter *et al.*, 1976).

These particles may be produced either by x-ray-induced polymerization in the case of methyl methacrylate (Kreuter and Speiser, 1976*a*; Kreuter *et al.*, 1976) or by base-catalyzed polymerization in the case of the cyanocrylates (Couvreur *et al.*, 1979). Polymerization in the presence of the antigen led to a partial incorporation of the antigen into the particles, while polymerization in absence of the antigen led to the adsorption of the antigen onto the particle surface (Kreuter and Speiser, 1976*b*). γ-Irradiation was chosen as one of the polymerization methods because it does not destroy the antigenicity of a number of antigens (Kreuter and Zehnder, 1978).

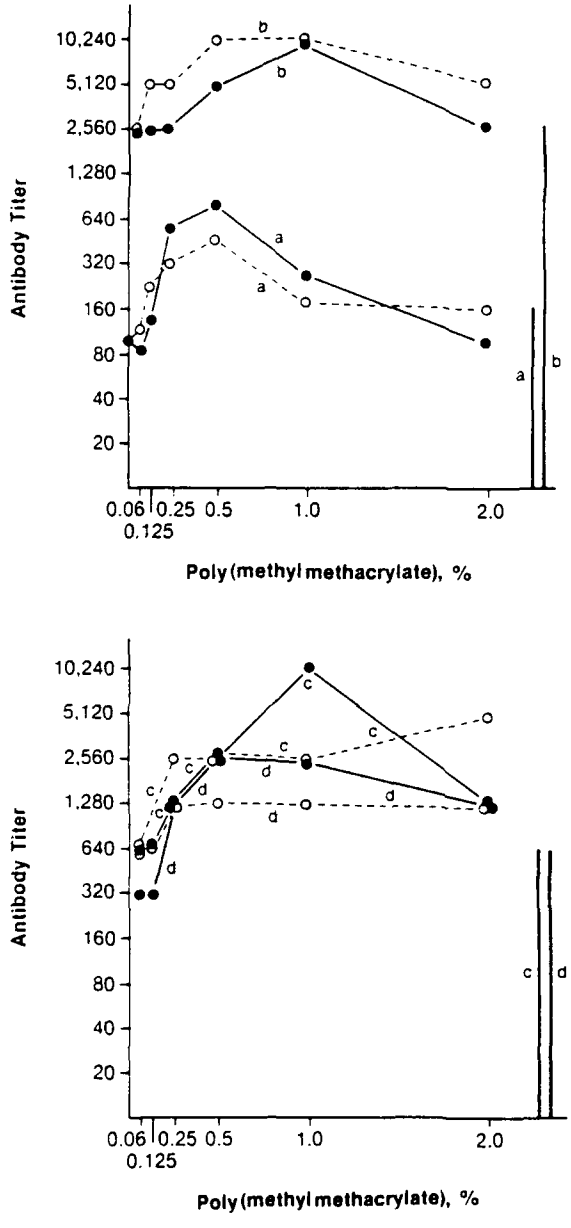

Figure 8. Influence of different contents of poly(methylmethacrylate) on the antibody response of mice after 28 (a), 36 (b), 64 (c), and 92 (d) days. Boosting occurred after 28 days with the same vaccine as was used in the primary vaccination. (-●-) Incorporation into poly(methyl methacrylate). (-○-) Adsorption onto poly(methylmethacrylate) (|) Adsorption onto 0.2% aluminum hydroxide.

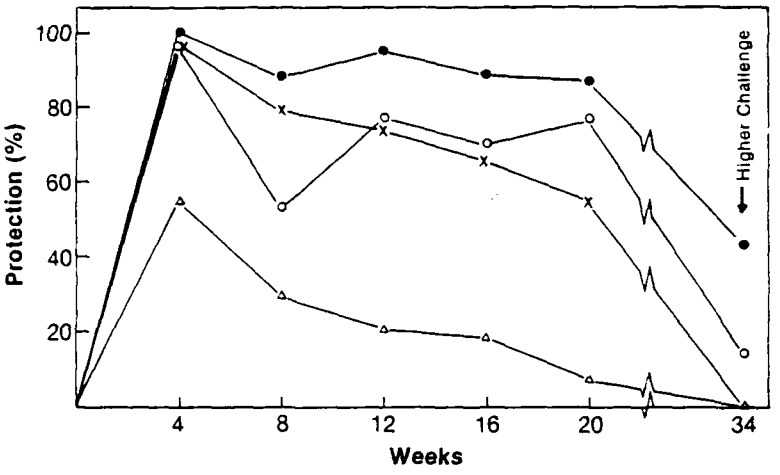

Figure 9. Protection against infection after challenge with 50 LD'_{50} of mouse-adapted influenza virus. Higher challenge = 250 LD_{50}. The vaccines contained the following adjuvants. (●) Incorporation into 0.5% poly(methylmethacrylate). (○) Adsorption onto 0.5% poly(methylmethacrylate). (x) Adsorption onto 0.2% aluminium hydroxide. (△) Fluid vaccine.

The vaccines with nanoparticles as adjuvants were tested by determination of the antibody response in guinea pigs (Langer, 1981; Kreuter and Speiser, 1976*a*) and mice (Langer, 1981; Kreuter and Speiser, 1976*a*; Kreuter and Zehnder, 1978) as well as by measurement of the protection of mice against infection with an LD_{50} of mouse-adapted live influenza virus (Kreuter and Liehl, 1978, 1981).

Poly(methylmethacrylate) nanoparticles obtained an optimal adjuvant effect at a concentration of 0.5%. This optimum was considerably more pronounced with incorporated influenza antigen than with the adsorbed product (Fig. 8). At optimal concentrations, this adjuvant yielded a better antibody response (Kreuter and Speiser, 1976*a*; Kreuter *et al.*, 1976; Kreuter and Liehl, 1981) and better protection (Kreuter and Liehl, 1981) than did aluminum hydroxide. This improvement in adjuvant effect was especially pronounced after extended times (Fig. 9). The use of the polymeric adjuvants did improve the storage stability of the vaccines at higher temperatures (Fig. 10).

C. Controlled Systemic Release of Interferon with Biodegradable Polymer

To achieve controlled release of an interferon over a longer time frame than was possible with liposomal formulations, biodegradable polymers of poly(*d,l*-lactide-co-glycolide) (PLGA) were employed. Such polymers have been used in the development of controlled-release systems for small peptide hormones, such as luteinizing hormone-releasing hormone (LH-RH) (Sanders *et al.*, 1984), but their application to larger more labile polypeptides requires quite different formulation techniques in order to avoid protein denaturation.

It was possible to prepare $rHuIFN_{\beta ser17}$ in PLGA polymer matrices with 100% incorporation of interferon and full retention of biologic activity by using novel formula-

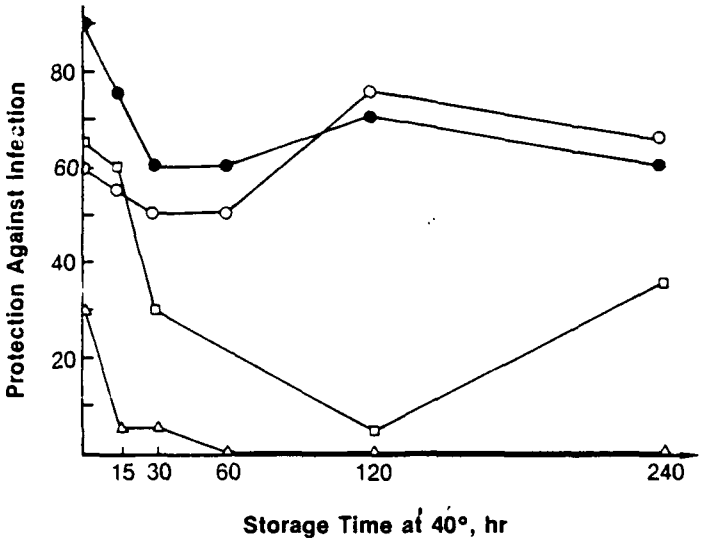

Figure 10. Stability of vaccines against heat inactivation, showing protection of mice against morbidity after immunization with vaccines that were stored at 40°C for different time periods. The vaccines contained the following adjuvants. (●) Incorporation into 0.5% poly(methyl methacrylate). (○) Adsorption onto 0.5% poly(methyl methacrylate). (x) Adsorption onto 0.2% aluminum hydroxide. (△) Fluid vaccine without adjuvants. The mice were challenged with 50 times the LD_{50} value of homologous mouse-adapted virus.

tion techniques (Schryver, van der Pas, and Eppstein, manuscript in preparation). The release profile of the interferon was determined over 3 months by quantitating the radioactivity remaining in a mouse at the site of SC implantation of a PLGA–interferon pellet or film (containing [^{125}I]-rHuIFN$_{\beta ser17}$ (Eppstein, 1986*a*). The release profile of the interferon was influenced both by the method of preparation as well as by the geometry of the final implant.

Release profiles were obtained over 2 months by employing different formulation methods as well as different final implant geometries. With some formulations, a very triphasic release profile was obtained, as has been observed with PLGA formulations of small peptides (Sanders *et al.*, 1984). Such a triphasic release profile is believed to represent first an augmented initial release rate of peptide as a result of diffusion from the surface of the PLGA implant or microspheres, followed by a relatively latent period of minimal drug release, while the polymer chains are gradually hydrolyzed to progressively shorter chain lengths, culminating with a high-release rate when the polymer has become hydrolyzed to sufficiently short chain lengths to become solubilized (Sanders *et al.*, 1984). However, by varying parameters of implant geometry, *in vivo* release profiles of interferon could be obtained that were fairly linear over 60–70 days. Extraction of the interferon from implants retrieved up to 5 weeks after implantation in the mouse showed that the rHuIFN$_{\beta ser17}$ retained remarkably good biologic (antiviral) activity. In fact, the PLGA formulation appeared to stabilize the interferon significantly, such that only a 0.3 $\log_{10}$ loss in biologic activity occurred after 5 weeks of implantation in the mouse. More than two $\log_{10}$ values of activity were lost when the same interferon (containing sta-

bilizers) was incubated at 37°C in buffer in a test tube (Eppstein, 1986*a*; Schryver, van der Pas, and Eppstein, manuscript in preparation).

Although sustained-release delivery systems have been shown to act as immunologic adjuvants for certain proteins (Amkraut and Martins, 1984), no antibody formation (neutralizing or non-neutralizing) was detected in the mouse with this human interferon–PLGA formulation. As the actual daily dose of interferon protein can be quite low in a sustained-release system, this may help circumvent the unwanted formation of antibodies against the exogenous interferon.

D. Treatment of Influenza Virus Infection with Squalene-in-Water Preparations

Studies were performed to investigate the ability of bacterial-derived immunomodulators, natural trehalose dimycolate (TDM), or synthetic muramyl dipeptide (MDP) to stimulate nonspecific resistance against respiratory viral infection by a well-defined aerosol model of influenza virus in mice (Table 5). The compounds were prepared in 1% squalene-in-water and adsorbed to the surface or mixed within the oil droplets. Outbred NMRI mice were pretreated with MDP, TDM, or a combination of MDP + TDM. A relatively long period (3–4 weeks) elapsed before aerosol infection with mouse-virulent A/PR/8/34 (H1N1) influenza virus. The results, summarized in Table 5, show that 150 μg MDP in saline was ineffective against a lower dose (LD_{80}) of influenza virus, whereas administration of MDP in 1% squalene-in-water preparation provided borderline protection. The increase in survival was not statistically significant. A dose of 75 μg of TDM in squalene-in-water preparation induced significant protection in 70% of treated mice.

The combination of 150 μg MDP plus 75 μg TDM afforded complete protection

Table 5. Effect of MDP and TDM on Aerogenic Influenza Viral Infection in Mice[a,b]

Pretreatment	Dose (μg)	% Surviving influenza infection	
		LD_{80}	LD_{100}
MDP–saline	150	30	0
MDP–1% squalene-in-water	150	50	0
	300	40	0
TDM–1% squalene-in-water	75	70	15
	150	70	10
MDP + TDM 1% squalene-in-water	150 + 75	100	91
	300 + 150	100	90
Formalinized influenza vaccine	200 HAU	100	100
1% squalene-in-water		20	0

[a] All treated by the IV route.

[b] Groups of 10–20 mice were pretreated 3 weeks before A/PR/8/34 influenza viral infection. Formalin-inactivated A/PR/8/34 influenza virus was used as specific vaccine.

against influenza virus infection (LD_{80}). When given alone, MDP and TDM were ineffective against a completely lethal infection (LD_{100}), even when higher doses of 300 μg or 150 μg, respectively, were used. The combination of MDP + TDM was consistently found to be efficacious against both low and high doses of influenza virus.

The efficacy of MDP + TDM combination was corroborated by tissue culture titration of the virus in the infected lungs. MDP + TDM-pretreated mice showed marked decreased levels of virus 72 hr after infection, and the clearance of detectable infectious virus occurred earlier in this group. Serum hemagglutination-inhibiting antibodies could be detected already on day 5 in the MDP + TDM-pretreated mice, but only on day 7 in the control group. It should be mentioned that all survivors of the pretreated group showed a long-lasting resistance against lethal rechallenge 6 months later, even if a heterologous influenza [A/Port Chalmers/1/73 (H3N2)] virus was used for rechallenge (Fig. 11).

Significant protection against influenza virus mortality was obtained with 6-0-acyl analogues and a ubiquinone derivative of MDP. A greater degree of protection was induced by the combination of TDM with lipophilic derivatives, like the seryl analogue of desmethyl-MDP (Table 6).

Cell walls of gram-negative bacteria contain endotoxic lipopolysaccharide, a potent stimulator of the immune system, even in nanogram quantities. Clinical application of endotoxin has been hampered, as it plays a major role in the pathophysiology of bacterial sepsis. Concerted efforts have been made to modify endotoxin for possible therapeutic use in humans. Investigations on endotoxic lipopolysaccharide have led to the identification of lipid A as an important immunopharmacologic constituent of the endotoxin molecule.

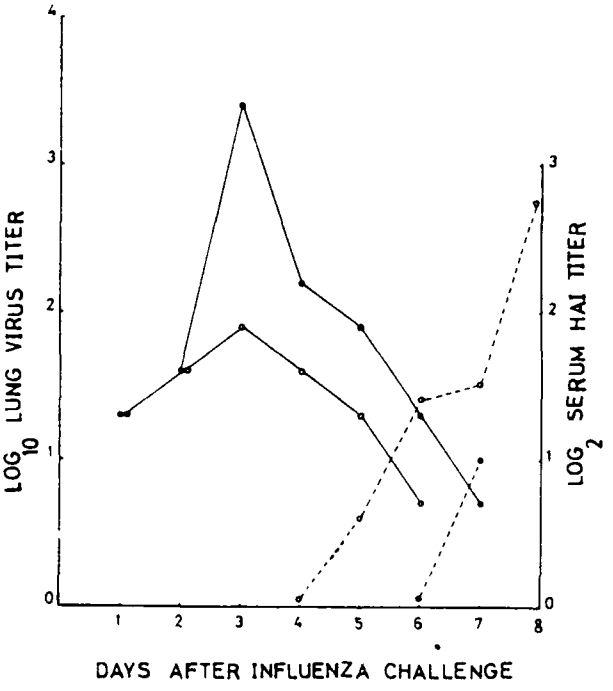

Figure 11. Reduction of A/PR/8/34 (HINI) influenza virus in the lungs of mice treated with 150 μg MDP and 75 μg TDM.

Table 6. MDP Analogues Effective against Influenza Viral Infection in Mice When Combined with TDM

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)
6-0-(2-Tetradecylhexadecanoyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (B30-MDP)
6-0-Stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (L18-MDP)
6-0-Isopentadecanoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (Iso.15-MDP)
2,3-Dimethoxy-5-methyl-6-(9'-carboxynonyl)-1,4-benzoquinone-*N*-acetylmuramyl-L-valyl-D-isoglutamine-methyl ester (QS-10-MDP-66)
N-Acetylmuramyl-L-seryl-D-isoglutamine
N-Acetyldesmethylmuramyl-L-seryl-D-isoglutamine
6-0-Succinyl-*N*-acetyldesmethylmuramyl-L-seryl-D-isoglutamine
6-0-Capryl-*N*-acetyldesmethylmuramyl-L-seryl-D-isoglutamine
N-Acetylmuramyl-L-α-aminobutyryl-D-isoglutamine
N-Acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine

Recently, selective reduction of the harmful toxicity and pyrogenicity of lipid A, while retaining its beneficial adjuvant property, have been achieved. Effective chemical treatment of toxic lipid A isolated from refined cell walls (CWS) of polysaccharide-deficient heptose-free Re mutant strains of *Salmonella typhimurium* or *Salmonella minnesota* yielded monophosphoryl lipid A (MPL), which was 1000-fold less toxic than endotoxin. The availability of nontoxic MPL has already led to clinical trials in human patients.

Combinations of MPL with CWS and TDM in squalene-in-water emulsions were investigated for their effect on influenza viral infection. The results of a representative experiment show that the combination of 100 μg of CWS with either 50 μg MPL or 50 μg TDM did not induce significant resistance. By contrast, the combination of 50 μg TDM with MPL afforded complete protection against lethal disease. Even amounts as low as 5 μg MPL combined with 50 μg TDM could induce significant resistance to the viral infection. Combination treatment with CWS, MPL, and TDM was effective when at least 50 μg TDM was used in the preparations (Table 7).

The efficacy of the MPL + TDM combination was checked by the titration of the virus present in the lungs. The resistance-stimulating MPL + TDM combinations significantly reduced lung viral titers, in contrast to the ineffective CWS + MPL combinations or the controls.

Natural killer (NK) cell activity of 3–4-week pretreated animals was not significantly different from controls at the time of infection. In contrast to NK cells, macrophages appear to be among the target cells for the immunopotentiating activity of MPL + TDM combination 2–4 weeks after pretreatment.

To elucidate the role of the macrophage in the resistance induced by MDP + TDM combination, animals pretreated 21 days earlier with the immunostimulants were given silica, dextran sulfate, and carrageenan, and 24 hr later, an aerosol of influenza virus. The results show that the resistance to influenza virus that was induced by MDP + TDM combination was abrogated by treatment with all three agents known to inhibit selectively or impair macrophage function *in vivo* (Table 8).

Phagocytosis stimulates oxidative metabolism of the phagocytic cells, which results in increased hexose monophosphate shunt activity and the generation of activated oxygen

Table 7. Protection against Influenza Viral Infection by Combination of MPL, TDM, and CW

Treatment[a]	Dose (μg)	% Survival	*p*
MPL	50	0	—
TDM	50	0	—
CWS	100	0	—
CWS + MPL	100 + 50	0	—
CWS + TDM	100 + 50	0	—
MPL + TDM	50 + 50	100	<0.001
	5 + 50	30	<0.05
	50 + 5	0	—
CWS + MPL + TDM	100 + 50 + 50	80	<0.001
	100 + 5 + 50	40	<0.02
	100 + 50 + 5	0	—
Vehicle control	—	0	—

[a] Groups of 10–20 mice were pretreated intravenously with various squalene-in-water emulsions 3 weeks before aerosol infection with A/PR/8/34 influenza virus.

metabolites, such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical with the emission of photons. The release of energy in the form of light (chemiluminescence) can be detected and measured. Spleen cells from animals pretreated with the combination of MDP + TDM exhibited markedly enhanced chemiluminescence activity in response to stimulation by *Staphylococcus aureus;* similar results were obtained with zymosan (Fig. 12).

The effects of lipophilic MDP on the morphology of murine macrophages were investigated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, alteration of the phagocytic function was determined by Fc receptor-mediated phagocytosis of ^{51}Cr-labeled sheep erythrocytes. Resident peritoneal macrophages cultured *in vitro* for 24 hr with 100 μg lipophilic MDP underwent a striking change in appearance. Rounding of macrophages and large cytoplasmic vacuoles were

Table 8. Abrogation of Effect of MDP and TDM on Aerogenic Influenza Viral Infection in Mice with Silica, Carrageenan, and Dextran Sulfate[a]

Pretreatment (150 + 75 μg)	Compound	Dose (mg)	% Survival	*p*
MDP + TDM	—	—	90	—
MDP + TDM	Silica	3	10	0.01
MDP + TDM	Carrageenan	1	10	0.01
MDP + TDM	Dextran sulfate	1	20	0.02

[a] Groups of 10 mice were pretreated with 1% squalene-in-water preparations 3 weeks before aerosol infection with A/PR/8/34 influenza virus. Anti-macrophage compounds were administered intravenously 24 hr before infection.

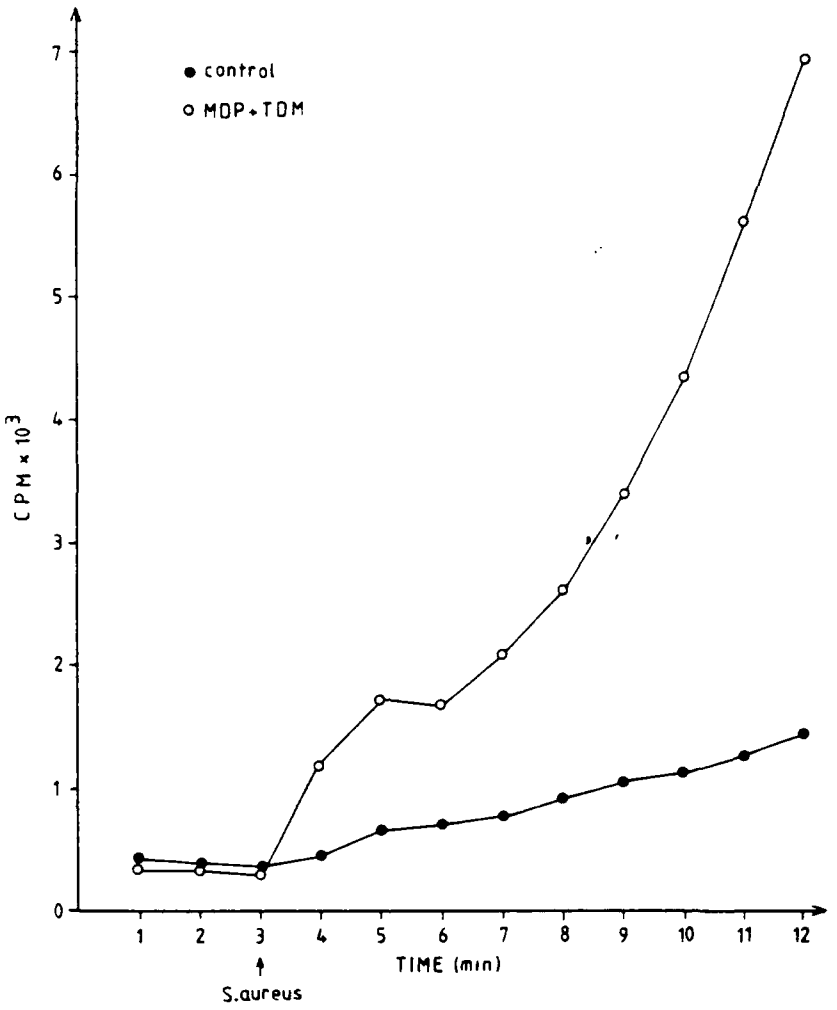

Figure 12. Enhancement of chemiluminescense activity of mouse spleen cells with MDP and TDM combination treatment.

observed. Figure 13 shows SEMs of untreated and MDP-treated macrophages. Untreated macrophages (Fig. 13A) showed spreading elongated forms with many ridges, microvilli, and thin pseudopods. By contrast, MDP-treated macrophages (Fig. 13B) showed rounding and extensive ruffling of the cell surface.

Transmission electron microscopy confirmed the morphologic changes. Large vacuoles were observed in the cytoplasm of macrophages treated with lipophilic MDP (Fig. 13B) but not in the untreated macrophages (Fig. 13A). The morphologic alterations in the treated macrophages could not be ascribed to nonspecific cytotoxic effects, since the viability of macrophages was not affected by lipophilic MDP, as shown by dye exclusion.

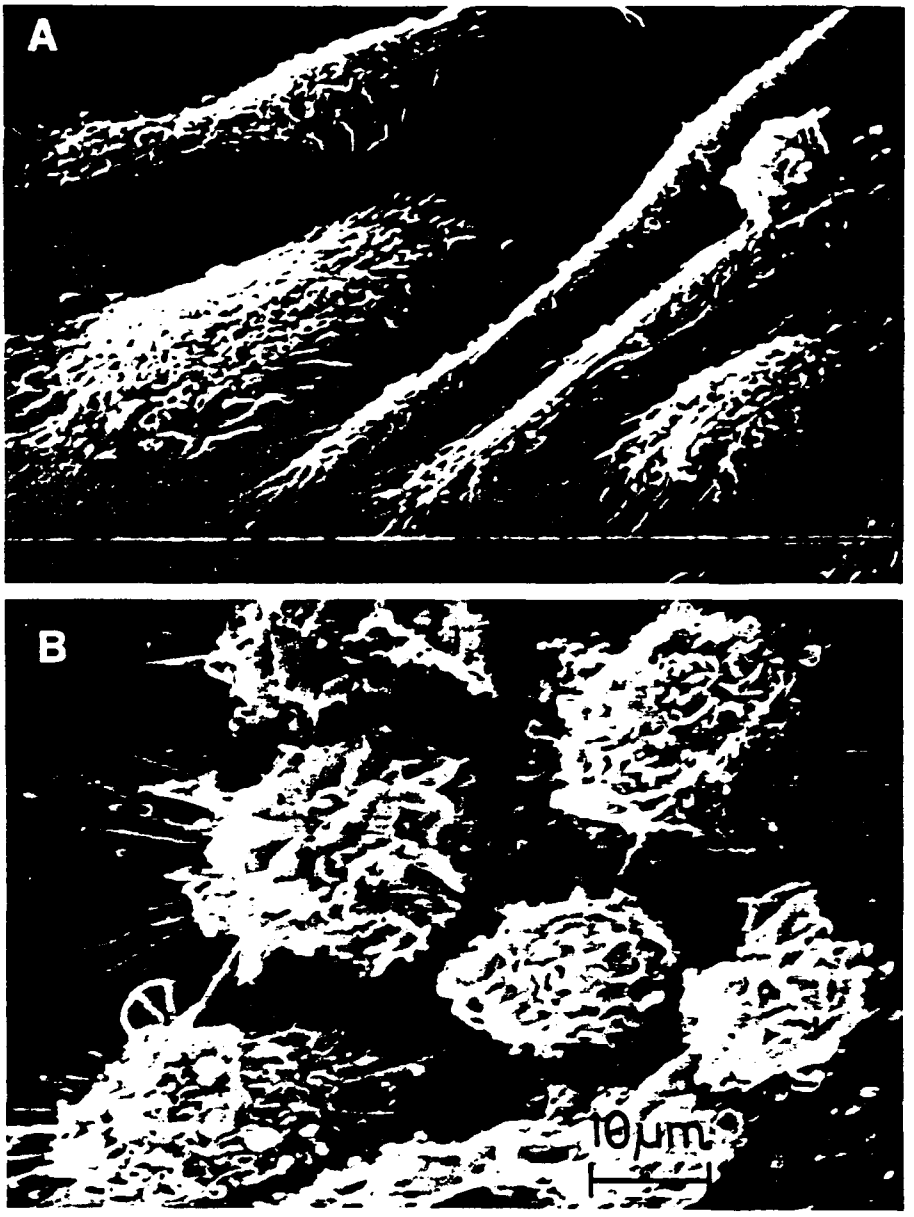

Figure 13. Scanning electron micrograph of untreated (A) and MDP-treated (B) mouse macrophages.

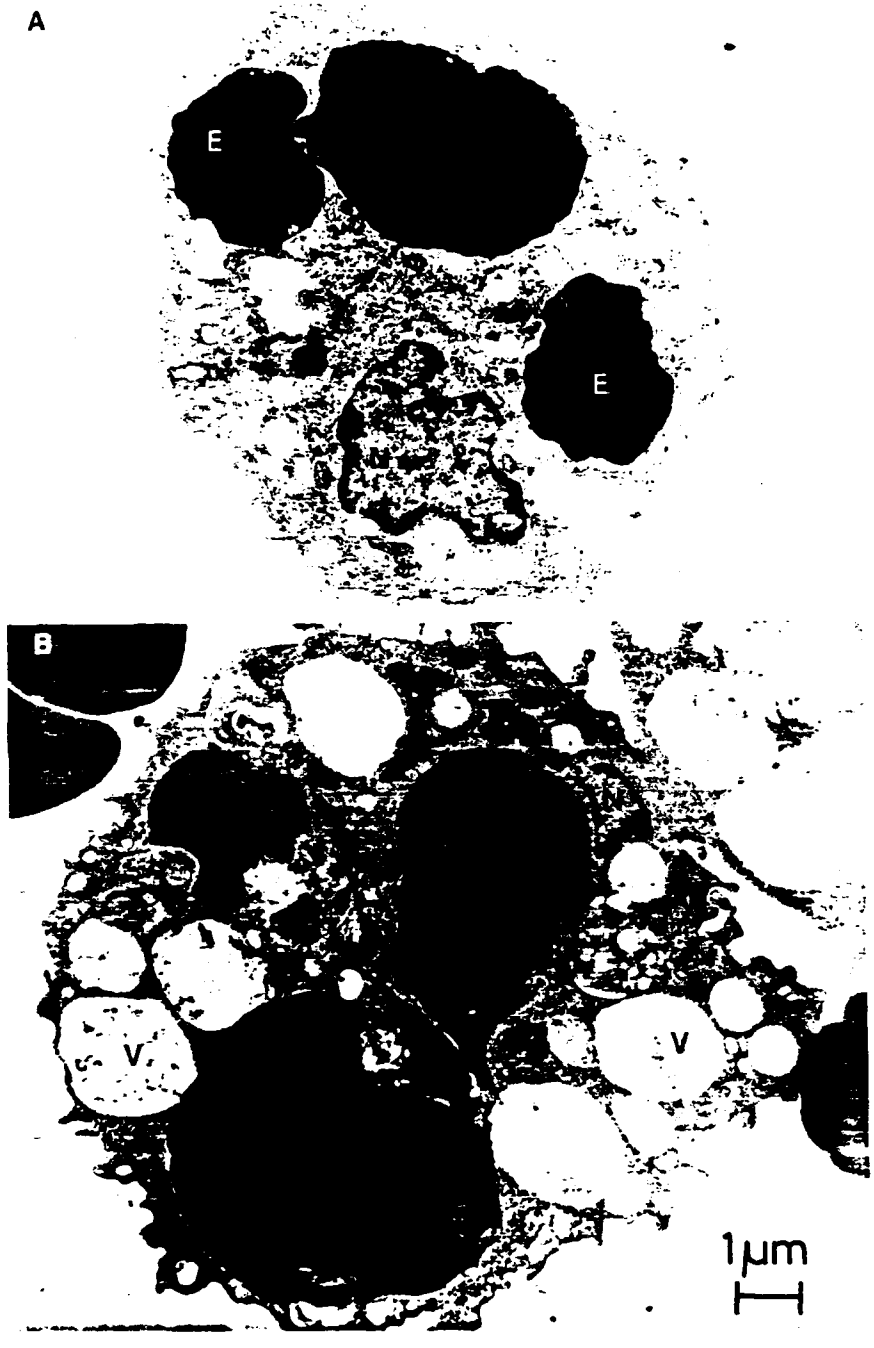

Figure 14. Transmission electron micrograph of untreated (A) and MDP-treated (B) mouse macrophages showing phagocytolysis of sheep erythrocytes.

Figure 15. Scanning electron microscopy sheep erythrocytes bound to MDP-treated macrophages.

The ability of treated and control macrophages for Fc receptor-mediated phagocytosis of ^{51}Cr-labeled sheep erythrocytes was tested. A large number of sheep erythrocytes were ingested by lipophilic MDP-treated macrophages, in contrast to the untreated controls, as shown in the TEMs presented in Fig. 14. SEM revealed many opsonized sheep erythrocytes bound to the treated macrophages (Fig. 15).

Thus, the combination of trehalose dimycolate with muramyldipeptides or monophosphoryl lipid A induces long-term resistance, and the mechanisms involved include macrophage activation. Stimulation of nonspecific host-defense mechanisms against viral infections by potent immunomodulators appears feasible.

V. CONCLUSION

Increased antiviral efficacy was achieved with liposomal drug delivery via endocytic phagocytosis or by localized sustained release. Although comparative delivery of the same compound with both systems and the assessment of their antiviral efficacy has not been done, direct delivery into cells is probably more efficient. Liposomal delivery of antiviral compounds into cells is currently limited to macrophages alone, but localized delivery with liposomes provides a viable alternative. Development of targeted delivery of a polymer–matrix–drug complex via receptor-mediated endocytic pinocytosis into cells other than reticuloendothelial origin also appears to be attainable. Furthermore, systemic

delivery with polymeric microspheres or with squalene-in-water has also been proved suitable in order to achieve the much-needed control of viral infections. The pitfall of these systems is that they cannot deliver antiviral agents into the CNS. For this purpose, new currently nonexisting technology will be required.

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